Version With Markings To Show Changes Made To Specification

In the direct MAP synthesis route with stepwise assembly of the desired peptide on the lysinyl core a peptide antigen having C to N orientation is obtained. Such a MAP may be useful for ELISA applications, where antigen recognition by an antibody is directed at the N-terminal or preferably close to the N-terminal, while recognition of the C-terminal may be hindered. This is e.g. the case with the dominant epitope of the Outer surface protein C (OspC) from Borrelia burgdorferi sensu lato causing Lyme borreliosis. Epitope mapping of OspC using sera from patients with neuroborreliosis led to identification of one single major immunodominant epitope within the C-terminal ten amino acid residues, the decapeptide PVVAESPKKP (Seq. ID 1). Peptide binding studies and alanine scanning revealed a critical role for the PKKP-sequence (Seq. ID 7) and in particular its terminal carboxyl group for the binding of IgM antibodies from patients with Lyme borreliosis. Thus substitution of the C-terminal proline or replacement of the carboxy group with a carboxamido group greatly reduced the ability of the peptides to compete with OspC19-207 for the binding of IgM antibodies in all five sera investigated. This significant fact clearly [exclude] excludes a MAP formed by stepwise synthesis on a lysine core as this results in C-terminal attachment to the core matrix.

From the above discussion it is clear that a system providing a choice between C-, and C- and N-terminal presentation of sequences, and at the same time being easily performed and yielding improved products suitable for various applications, is needed.

SUMMARY OF THE INVENTION

The present invention provides novel methods for preparing LPAs enabling presentation of desired sequence(s). In a further aspect, the present invention relates to a method for preparing LPAs enabling presentation of desired sequence(s) and chemical moie-

ties.

In another aspect, the present invention relates to an LPA obtainable by the method.

In yet a further aspect, the present invention relates to immunological compositions and vaccines, as well as a method of generating antibodies in an animal, including a human being.

Furthermore, the present invention relates to the use of such LPAs in various applications and kits.

The present invention is described in detail below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the MALDI-TOF MS spectrum of the crude product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 2 shows the MALDI-TOF MS spectrum of the crude product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 3 shows the HPLC chromatogram of crude H-ProValValAlaGluSer-ProLysLysPro-OH (also denoted H-Seq. ID 1-OH), cf. Example 3.

Figure 4 shows the HPLC chromatogram of crude $NH_2CH(CH_2CO\text{-}ProValVal\text{-}AlaGluSerProLysLysPro\text{-}OH)_2$ (also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$) [LPA-III] of Example 3.

Figure 5 shows the HPLC chromatogram of the HPLC purified product $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ [(] (also denoted

and strong antibody response in the majority of patients. These are the B. burgdorferi flagellum and outer surface protein C (OspC). Whereas the performance of EIAs (enzyme immuno assays) using purified native B. burgdorferi flagellum is well documented, the reported experience with OspC EIAs is still limited.

WO 97/42221 (ref. 25) discloses that the 4 terminal amino acids Pro-Lys-Lys-Pro (Seq. ID 7) are essential in the immune reactivity between sera from patients suffering from early borreliosis and various OspC derivatives. In order to obtain an effective diagnostic agent a polypeptide fragment which contains carboxyterminally a peptide with the general formula A^5-A^4-A^3-A^2-A^1, where A^1, and A^4, independently from each other, designate a residue of an amino acid, wherein a nitrogen atom capable of forming part of a peptide bond is part of a ring structure; A^2 and A^3, independently from each other, designate residues or a positively charged or polar amino acid; and A^5 designates residues of any amino acid, such that the peptide of the formula above has a degree of sequence identity of at least 60% with the amino acid residue subsequence of Ser-Pro-Lys-Lys-Pro (Seq. ID 8) is preferred. Such polypeptide fragments may advantageously be used in preparing a multimeric structure according to the invention. Particular preferred OspC carboxyterminal peptides are Val-Ala-Glu-Ser-Pro-Lys-Lys-Pro (Seq. ID 9), Val-Val-Ala-Glu-Ser-Pro-Lys-Lys-Pro (Seq. ID 10), and Pro-Val-Val-Ala-Glu-Ser-Pro-Lys-Lys-Pro (Seq. ID 1).

In one embodiment of the present method, at least one of the sequences is derived from a sequence, wherein the C-terminal amino acids are important for an immune response.

In another embodiment of the present method, the desired sequence is derived from the OspC protein of Borrelia burgdorferi, or is a homologous sequence capable of reacting with anti-OspC antibodies,

or is capable of provoking an immune response. In particular, the LPA obtained provides a C-terminal presentation of the C-terminal sequence Pro-Lys-Lys-Pro (Seq. ID 7) of OspC.

In accordance with the invention, the presentation of a carboxyterminal OspC related peptide as described above can be combined with the presentation of another sequence relevant for diagnosing Lyme borreliosis. In particular, for early diagnosis of Lyme borreliosis sequences comprising relevant epitopes from the flagellum are relevant. Other relevant sequences might be derived from OspA, OspB, OspD, OspE, OspF, Erp proteins, BmpA (P39), P100, P35, P37 and DbpA. In particular, sequences derived from OspC, the flagellum, BmpA, (P39) or P100 are suitable sequences for diagnostic uses. Sequences derived from OspA, OspC or DbpA are suitable vaccine candidates.

In yet another embodiment, the desired sequence is derived from the flagellum of Borrelia burgdorferi or is a sequence homologous thereto capable of reacting with anti-flagellum antibodies.

In yet another embodiment of the present invention, the LPA providing C-terminal presentation of sequences derived from OspC of Borrelia burgdorferi further comprises desired sequence(s) derived from the flagellum of Borrelia burgdorferi.

In one embodiment of the present invention, the desired sequence is derived from Mycobacterium tuberculosis. In a further embodiment, the LPA of the invention provides C-terminal presentation of the desired sequences of OspC derived from Borrelia burgdorferi, and N-terminal presentation of desired sequence(s) derived from Mycobacterium tuberculosis. In particular, the desired sequence derived from M. tuberculosis may comprise the sequence of amino acids number 51 to 70 of ESAT-6 (ESAT-6, 51-70 sequence protein)

(Seq. ID 7) of OspC.

Examples of suitable LPAs are

[LPA I]: $FmocN(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ also denoted $FmocN(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$,

[LPA II]: $biotin\text{-}NH(CH_2)_5CON(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ also denoted $biotin\text{-}NH(CH_2)_5CON(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$,

[LPA III]: $NH_2CH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ also denoted $NH_2CH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$, and

[LPA IV]: $H\text{-}Lys\text{-}NHCH(CH_2CO\text{-}ProValValAlaGluSerProLysLysPro\text{-}OH)_2$ also denoted $H\text{-}Lys\text{-}NHCH(CH_2CO\text{-}Seq.\ ID\ 1\text{-}OH)_2$.

In another embodiment of the LPA of the present invention, the desired sequence is derived from Mycobacterium tuberculosis. In a further embodiment of the LPA, C-terminal presentation of the desired sequences derived from OspC of Borrelia burgdorferi, and N-terminal presentation of desired sequence(s) derived from Mycobacterium tuberculosis are provided.

In particular, the LPA providing C-terminal presentation of the C-terminal Pro-Lys-Lys-Pro (Seq. ID 7) of OspC of Borrelia burgdorferi, and N-terminal presentation of ESAT-6 sequences from M. tuberculosis may be selected from

[LPA-V]: $(HO\text{-}ProLysLysProSerGluAlaValValPro\text{-}COCH_2)_2CH\text{-}NH\text{-}Lys\text{-}(GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr\text{-}H)_2$ also denoted $(HO\text{-}Seq.\ ID\ 1\text{-}COCH_2)_2CH\text{-}NH\text{-}Lys(Seq.\ ID\ 2\text{-}H)_2$, and

[LPA-VI]: $(HO\text{-}ProLysLysProSerGluAlaValValPro\text{-}COCH_2)_2N\text{-}Lys(AlaSer\text{-}$

administration.

In another aspect, the present invention relates to a method for generating antibodies in an animal, including a human being, which method comprises administering to said mammal an antibody-generating amount of an LPA or an immunological composition as defined above.

Furthermore, the present invention relates to the use of an LPA as defined above for preparing a pharmaceutical composition for the treatment, alleviation, or prophylaxis of diseases caused by viruses, bacteria, toxins, allergens, autoimmune system-related compounds, cancer related compounds, cell adhesion molecules, neurotropic factors, fungi or parasites.

Formulation of vaccines

Although it is preferred to insert a whole B- or T-cell epitope, it may in some cases be advantageous to insert a sequence comprising both the epitope as well as flanking regions from the protein from which the epitope is derived. The amino acids of a given epitope may be located near each other when the sequence is in the three-dimensional structure, but distant when the sequence is denatured. The sequence may be composed of the amino acids actually forming the epitope in the three-dimensional structure omitting all or part of the other amino acids.

[According to the invention the LPAs may be used in vaccines against infections with pathogenic agents as described above. Strategies in formulation development of vaccines based on the products obtained by the method of the present invention generally correspond to formulation strategies for any other protein-based drug product. Potential problems and the guidance required to] According to the invention the LPAs may be used in vaccines against infections with pathogenic agents as described above. Strategies in formulation development of vaccines based on the products obtained by the method of the present invention generally correspond to formulation strategies for any other protein-based drug product. Potential problems and the guidance required to

[overcome these problems are dealt with in several textbooks. The use of an adjuvant, e.g., aluminium hydroxide, aluminium phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analogue, or some of the more recent developments in vaccine adjuvants such as biodegradable microparticles and Iscom's is a formulation challenge familiar to a pharmaceutical scientist working in this area. Usually vaccines require the use of adjuvants. According to the invention the modified human TNFa molecules can be formulated with such appropriate adjuvants, e.g. aluminium phosphate (Adju-Phos) or other alternative adjuvants such as aluminium hydroxide, calcium phosphate, muramyl dipeptide analogs, Iscom's or other known adjuvants used in mammalian vaccines.]

overcome these problems are dealt with in several textbooks. The use of an adjuvant, e.g., aluminium hydroxide, aluminium phosphate (Adju-Phos), calcium phosphate, muramyl dipeptide analogue, or some of the more recent developments in vaccine adjuvants such as biodegradable microparticles and Iscom's is a formulation challenge familiar to a pharmaceutical scientist working in this area. Usually vaccines require the use of adjuvants. According to the invention the modified human TNFa molecules can be formulated with such appropriate adjuvants, e.g. aluminium phosphate (Adju-Phos) or other alternative adjuvants such as aluminium hydroxide, calcium phosphate, muramyl dipeptide analogs, Iscom's or other known adjuvants used in mammalian vaccines.

Preparation of the vaccines according to the invention which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by US 4 608 251 (ref. 27), US 4 601 903 (ref. 28), US 4 599 231 (ref. 29), US 4 599 230 (ref. 30), US 4 596 792 (ref. 31), and US 4 578 770 (ref. 32), all incorporated herein by reference. Typically, such vaccines are pre-prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of

invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant, but also QuilA and RIBI are interesting possibilities. Further possibilities are monophosphoryl lipid A (MPL), and muramyl dipeptide (MDP). [Other suitable adjuvants are calcium phosphate, and muramyl dipeptide analogue. Some of the more recent developments in vaccine adjuvants such as biodegradable microparticles and Iscom's may also suitably be used.] Other suitable adjuvants are calcium phosphate, and muramyl dipeptide analogue. Some of the more recent developments in vaccine adjuvants such as biodegradable microparticles and Iscom's may also suitably be used.

Another highly interesting possibility of achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (which is hereby incorporated by reference). In brief, the presentation of a relevant antigen can be enhanced by conjugating such antigen to antibodies (or antigen binding antibody fragments) against the Fcγ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-FcγRI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of immune modulating substances such as lymphokines (e.g. IFN-γ, IL-2 and IL-12) or synthetic IFN-γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

In many instances, it will be necessary to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain the desired levels of protective immunity.

One reason for admixing the LPAs of the invention with an adjuvant is to effectively activate a cellular immune response.

(0.24 ml, 3.36 mmol) was added and the mixture was refluxed for 6 hours. After continued stirring over night, the mixture was concentrated to give an oil. Dissolution in ethyl acetate (5 ml) was followed by neutralisation with citric acid (10%, 3x5 ml). The organic phase was washed with water, dried over $MgSO_4$ and finally concentrated *in vacuo*. Re-crystallisation from the ethyl acetate gave 180 mg (63%). M.p. 213-214°C. FAB-MS: Calc. For $C_{13}H_{19}NO_4$, 253.3 found 254.0.

EXAMPLE 1

Synthesis of FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted FmocN(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin and FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted FmocN(CH_2CO-Seq. ID 1-OH)$_2$ (IDA-synthesis of Seq. ID 1)

Product: FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted FmocN(CH_2CO-Seq. ID 1-OH)$_2$ [LPA-I]

The antigenic C-terminal sequence of OspC from Borrelia burgdorferi ProValValAlaGluSerProLysLysPro (Seq. ID 1) was assembled on chlorotrityl resin (s = 0.8 mmol/g) as described above and tested for homogeneity. The peptide moiety ProValValAlaGluSerProLysLysPro (Seq. ID 1) has a molecular weight of 1035 Dalton and a corrected substitution of 0.44 is used for samples of the peptide-resin. 100 mg of the peptide-resin was suspended in DMF and deprotected with 20% piperidine-DMF. 0.55 eq Fmoc-iminodiacetic acid (i.e. 10% excess; 0.1x1.1x05x0.44x355 = 8.6 mg; Mw 355), 3.3 eq TBTU (0.1x1.1x3x0.44x321 = 46.6 mg; i.e. 3 times excess compared to the amount carboxy groups on Fmoc-iminodiacetic acid), 3.3 eq Hobt (0.1x1.1x3x0.44x135 = 19.6) and 4.95 eq DIEA (0.1x1.1x4.5x0.44x174 = 38 ml; standard amount of DIEA is 1.5xTBTU-eq.) was added to the resin and left overnight. Excess reagents were removed, the resin was washed with DMF, followed by ether and dried. The resulting

dimeric compound, FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted FmocN(CH_2CO-Seq. ID 1-OH)$_2$, was cleaved from the resin with 95% aqueous TFA. MALDI TOF MS: Calculated for $C_{115}H_{177}N_{25}O_{32}$, 2421.8; found 2423.2. The product contains only a small amount of the side-product FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)CH_2COOH also denoted FmocN(CH_2CO-Seq. ID 1-OH)CH_2COOH (Calculated for $C_{67}H_{97}N_{13}O_{19}$, 1388.6; found 1389.0) which is essentially absent upon repeated coupling.

Example 2

Synthesis of biotin-NH(CH_2)$_5$CON(CH_2CO-ProValValAlaGluSer-ProLysLysPro-OH)$_2$ also denoted biotin-NH(CH_2)$_5$CON(CH_2CO-Seq. ID 1-OH)$_2$ (IDA derivative of Seq. ID 1 extended with 6-amino-hexanoic acid and biotinylated)

Product: biotin-NH(CH_2)$_5$CON(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted [Biotin-NH(CH_2)$_5$CON(CH_2CO-Seq. ID 1-OH)$_2$] biotin-NH(CH_2)$_5$CON(CH_2CO-Seq. ID 1-OH)$_2$ [LPA-II]

FmocN(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted FmocN(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin was deprotected with 20% piperidine-DMF and coupled with Fmoc-6-amino-hexanoic acid activated with TBTU. The coupling was followed by exhaustive acetylation with acetic anhydride. After deprotection with 20% piperidine-DMF, biotin ($C_{10}H_{16}N_2O_3S$) was coupled, activated with TBTU. The product was cleaved from the resin with 95% aqueous TFA. MALDI TOF MS analysis showed the target compound as the major product (calculated for $C_{116}H_{192}N_{28}O_{33}S$, 2539.0; found 2536.3) containing some side-product, biotin-NH(CH_2)$_5$CON(CH_2CO-ProValValAla-GluSerProLysLysPro-OH)CH_2COOH also denoted biotin-NH(CH_2)$_5$CON(CH_2CO-Seq. ID 1-OH)CH_2COOH (calculated for $C_{68}H_{112}N_{16}O_{20}S$, 1505.8; found 1504.1).

EXAMPLE 3

Synthesis of FmocNHCH(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted FmocNHCH(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin and NH_2CH(CH_2CO-ProValValAlaGluS rProLysLysPro-

$OH)_2$ also denoted $NH_2CH(CH_2CO$-Seq. ID 1-$OH)_2$ (AGA-3-derivative of Seq. ID 1)

Product: $NH_2CH(CH_2CO$-ProValValAlaGluSerProLysLysPro-$OH)_2$ also denoted $NH_2CH(CH_2CO$-Seq. ID 1-$OH)_2$ [LPA III]

The resin derivative was assembled in the same way as described above for the iminodiacetic acid product using N-(Flouren-9-ylmethoxycarbonyl)-3-aminoglutaric acid. The resulting Fmoc-$NHCH(CH_2CO$-ProValValAlaGluSerProLysLysPro$)_2$-chlorotrityl resin also denoted Fmoc-$NHCH(CH_2CO$-Seq. ID 1$)_2$-chlorotrityl resin was deprotected with 20% piperidine-DMF and $NH_2CH(CH_2CO$-ProValValAlaGluSerProLysLysPro-$OH)_2$ also denoted $NH_2CH(CH_2CO$-Seq. ID 1-$OH)_2$ was obtained by cleavage from the resin with 95% aqueous TFA. MALDI-TOF MS: Calculated for $C_{101}H_{169}N_{25}O_{30}$, 2213.6; found 2215.2 (Figures 1 and 2). The higher molecular ions are the sodium (2238.0) and potassium ion adducts (2254.3) respectively. The product contains only a very small amount of lower molecular weight products as evidenced from the spectra. HPLC of crude H-ProValValAlaGluSerProLysLysPro-OH also denoted H-Seq. ID 1-OH and crude $NH_2CH(CH_2CO$-ProValVal[-]AlaGluSerProLysLysPro-$OH)_2$ also denoted $NH_2CH(CH_2CO$-Seq. ID 1-$OH)_2$ is shown in Figures 3 and 4, respectively. The HPLC of the HPLC-purified LPA-III is shown in Figure 5.

Example 4

Synthesis of Fmoc-Lys(Fmoc)-$NHCH(CH_2CO$-ProValValAlaGluSerProLysLysPro$)_2$-chlorotrityl resin also denoted Fmoc-Lys(Fmoc)-$NHCH(CH_2CO$-Seq. ID 1$)_2$-chlorotrityl resin and H-Lys-$NHCH(CH_2CO$-ProValValAlaGluSerProLysLysPro-$OH)_2$ also denoted H-Lys-$NHCH(CH_2CO$-Seq ID 1-$OH)_2$ (AGA-3-derivative of Seq. ID 1 extended with di-Fmoc-lysine)

Product: H-Lys-$NHCH(CH_2CO$-ProValValAlaGluSerProLysLysPro-$OH)_2$ also denoted H-Lys-$NHCH(CH_2CO$-Seq. ID 1-$OH)_2$ [LPA IV]

The above synthesised $NH_2CH(CH_2CO$-ProValValAlaGluSerProLysLysPro$)_2$-chlorotrityl resin also denoted $NH_2CH(CH_2CO$-Seq. ID 1$)_2$-chlorotrityl resin was coupled with di-Fmoc-lysine in 3 times

excess with TBTU as coupling agent. After Fmoc-deprotection with 20% piperidine-DMF, H-Lys-NHCH(CH_2CO-ProValValAlaGluSerProLysLysPro-OH)$_2$ also denoted H-Lys-NHCH(CH_2CO-Seq. ID 1-OH)$_2$ was cleaved from the resin with 95% aqueous TFA and precipitated with ether. MALDI TOF MS analysis: Calculated for $C_{107}H_{181}N_{27}O_{31}$, 2341.8; found 2343.2 (Figure 6).

Example 5

Synthesis of (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$CH-NH-Lys-(GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr-H)$_2$ also denoted (HO-Seq. ID 1-COCH$_2$)$_2$CH-NH-Lys-(Seq. ID 2-H)$_2$ (AGA-3-derivative of Seq. ID 1 extended with bis-ESTAT-6, 51-70 sequence (Seq. ID 2), on lysine)

Product (HO-ProLysLysProSerGluAlaValValPro-COCH$_2$)$_2$CH-NH-Lys-(GlnLeuAlaAsnAsnLeuGluThrAlaThrAlaAspTrpLysGlnGlnValGlyGlnTyr-H)$_2$ also denoted (HO-Seq. ID 1-COCH$_2$)$_2$CH-NH-Lys-(Seq. ID 2-H)$_2$ [LPA V]

FmocNCH(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted FmocNCH(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin (55 mg; s = 0.44), was deprotected with 20% piperidine-DMF and coupled with Fmoc-Lys(Fmoc)-OH activated with TBTU to give the above Fmoc-Lys(Fmoc)-NHCH(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted Fmoc-Lys(Fmoc)-NHCH(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin. After deprotection of Fmoc-groups the amino acids according to the sequence (ESAT-6, 51-70) were coupled as the Fmoc protected amino acids using HOAt and DIC as coupling agents in 4 times excess with ≥2 h couplings followed by a double coupling of ≥30 min. After completed assembly of the peptide chains, the peptide-resin was washed with DMF and diethyl ether and dried in vacuo. The assembled product was cleaved from the resin with 95% aqueous TFA and 5% triisopropylsilane and precipitated by addition of ether, redissolved in acetic acid/water and lyophilised to give 2 mg of the target product $C_{305}H_{481}N_{83}O_{97}$ (Mw 6862.7). HPLC showed a major peak. The product was analysed for homogeneity by amino acid analysis. Alanine and leucine was used for comparison giving 3941

and 1937 pmol respectively corresponding to a Ala/Leu ratio of 2.04 (exp 2.00). A double analysis similarly gave 2.03. The product was analysed by N-terminal amino acid sequencing and all 20 amino acids according to the ESAT sequence (Seq. ID 2) were recorded. The Borrelia OspC sequence (Seq. ID 1) is N-terminally coupled and consequently not sequenced.

EXAMPLE 6

Synthesis of (HO-ProLysLysProSerGluAlaValValPro-$COCH_2$)$_2$N-Lys-(AlaSerAlaAlaAlaGluIleGlyAlaPheAsnTrpGlnGlnGluThrMet-H)$_2$ also denoted (HO-Seq ID 1-$COCH_2$)$_2$N-Lys-(Seq. ID 3-H)$_2$ (IDA-derivative of Seq. ID 1 extended with Seq. ID 3 on lysine)

Product: (HO-ProLysLysProSerGluAlaValValPro-$COCH_2$)$_2$N-Lys-(AlaSerAlaAlaAlaGluIleGlyAlaPheAsnTrpGlnGlnGluThrMet-H)$_2$ also denoted (HO-Seq. ID 1- $COCH_2$)$_2$N-Lys(Seq. ID 3-H)$_2$ [LPA-VI]

Fmoc(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted Fmoc(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin (55 mg; S = 0.44), was deprotected with 20% piperidine-DMF and coupled with Fmoc-Lys(Fmoc)-OH activated with TBTU to give Fmoc-Lys(Fmoc)-N(CH_2CO-ProValValAlaGluSerProLysLysPro)$_2$-chlorotrityl resin also denoted Fmoc-Lys(Fmoc)-N(CH_2CO-Seq. ID 1)$_2$-chlorotrityl resin. After deprotection of Fmoc-groups with 20% piperidine-DMF the amino acids according to the ESAT-6, 1-17 sequence were coupled as the Fmoc protected amino acids using HOAt and DIC as coupling agents in 4 times excess with ≥2 h coupling followed by a double coupling of ≥30 min. After completed assembly of the peptide chains, the peptide-resin was washed with DMF and diethyl ether and dried in vacuo. The product was cleaved from the resin with 95% TFA and 5% triisopropylsilane and precipitated by addition of ether, redissolved in acetic acid/water and lyophilised to give 8.7 mg of the target product. MALDI TOF MS analysis: Calculated for $C_{264}H_{409}N_{69}O_{83}S_2$, 5941.7; found 5944.7. The product was analysed for homogeneity by amino acid analysis. Proline and valine from the

Borrelia pepC10 sequence was used for comparison with Gly from the ESAT-6, 1-17 sequence. Amino acid analysis gave 5044, 2823 and 1408 pmol for Pro, Val and Gly respectively giving a Gly/Pro ratio of 0.28 (exp. 0.33) and a Gly/Val ratio of 0.50 (exp. 0.500). A double analysis similarly gave 0.27 and 0.55. The product was analysed by N-terminal amino acid sequencing and all 17 amino acids according to the ESAT-6, 1-17 sequence (Seq. ID 3) were recorded. The Borrelia OspC sequence (Seq. ID 1) is N-terminally coupled and consequently not sequenced.

EXAMPLE 7

Synthesis of $CH_2(CH_2CO$-β-Ala-β-AlaLysGluProAsnLysGlyValAsnPro-AspGluValβAla$)_2$ also denoted $CH_2(CH_2CO$-β-Ala-β-Ala-Seq. ID 4-β-Ala$)_2$, (Glutaric acid-derivative, Fmoc-β-Ala and Dde-β-Ala synthesis of Seq. ID 4)

Product: $CH_2(CH_2CO$-β-Ala-β-AlaLysGluProAsnLysGlyValAsnPro-AspGluValβAla$)_2$ also denoted $CH_2(CH_2CO$-β-Ala-β-Ala-Seq. ID 4-β-Ala$)_2$ [LPA VII]

The β-Ala-β-AlaLysGluProAsnLysGlyValAsnProAspGluValβ-Ala sequence also denoted β-Ala-β-Ala-Seq. ID 4-β-Ala (LysGluProAsnLysGlyVal-AsnProAspGluVal (Seq. ID 4) of *Chlamydia trachomatis* DnaK 357-368) was assembled on chlorotrityl resin and on TentaGel S NH_2 resin with HMPA linker using standard coupling procedures with PyAOP 1.1 eq. and DIEA 2.2 eq. as coupling agents in DMF. In both cases the N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and subsequently coupled with a mixture of Fmoc-β-Ala and Dde-β-Ala to give an orthogonal 1:1 Fmoc/Dde-protection of the N-terminus resin-peptide chains. This was achieved by coupling with 0.75 eq. Fmoc-β-Ala and 0.25 eq. Dde-β-Ala using 1.1 eq. PyAOP and 2.2 eq. DIEA in NMP. The Fmoc-protecting group was deprotected with 20% piperidine-DMF followed by coupling with excess glutartic acid (6 eq.) and DIEA (12 eq.) in NMP over night. The remaining N-

terminal Dde-α-protecting groups were deprotected with 2% hydrazine in NMP for 10 minutes, followed by washing with 5% DIEA in NMP. Assembly was then achieved over night by activation with 6 eq. PyAOP and 12 eq. DIEA in NMP. The resulting product was cleaved from the resin with TFA-H_2O-TIS (92.5:5:2.5, vol/vol/vol). A single product with the correct mass was obtained with both resins. MALDI TOF MS: Calc. for $C_{135}H_{218}N_{38}O_{50}$, 3173.4; found 3169.7 (trityl-resin). Figure 9 shows the MALDI-TOF MS spectrum of the product.

EXAMPLE 8

Synthesis of $HC(CH_2CO$-LysGluProAsnLysGlyValAsnProAspGluValβAla$)_2COOH$ also denoted $HC(CH_2CO$-Seq. ID 4-βAla$)_2COOH$ (TCA-derivative, $CH(CH_2COOH)_2COOH$ synthesis of Seq. ID 4)

Product: $HC(CH_2CO$-LysGluProAsnLysGlyValAsnProAspGluValβAla$)_2COOH$ also denoted $HC(CH_2CO$-Seq. ID 4-βAla$)_2COOH$ [LPA VIII]

The LysGluProAsnLysGlyValAsnProAspGluValβAla sequence also denoted Seq. ID 4-βAla was assembled on TentaGel S NH_2 resin with HMPA linker (s = 0.19 mmol/g; 0.02 g) using standard coupling procedures with 3 eq. DIC and 3 eq. HOAt as coupling agents in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 eq. of $CH(CH_2COOH)_2COOH$, 1.65 eq. PyAOP and 3.3 eq DIEA in DMF over night. The resulting product was cleaved from the resin with TFA-H_2O-TIS-EDT (90:5:2.5:2.5, vol/vol/vol/vol) and submitted to gel filtration on G-25 Sephadex with 1% AcOH as eluent. The resulting fractions was lyophilysed to give 2 mg of the pure main product, $HC(CH_2CO$-LysGluProAsnLysGlyValAsnProAspGluValβAla$)_2COOH$ also denoted $HC(CH_2CO$-Seq. ID 4-βAla$)_2COOH$ (approx. 20% yield). MALDI TOF MS: Calc. for $C_{124}H_{198}N_{34}O_{48}$, 2933.1; found 2932.6. Figure 10 shows the MALDI-TOF MS spectrum for the product, and Figure 11 shows the HPLC chromatogram.

EXAMPLE 9

Synthesis of Fmoc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$, also denoted Fmoc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ (AGA-3-derivative of angiotensin-I amide, Fmoc-3-AGA synthesis of Seq. ID 5)

Product: Fmoc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Fmoc-NHCH-(CH_2CO-Seq. ID 5-NH_2)$_2$ [LPA IX]

The peptide sequence AspArgValTyrIleHisProPheHisLeu (Seq. ID 5) was assembled on TentaGel S RAM resin using standard coupling procedures with DIC and HOAt as coupling agent in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 eq. Fmoc-AGA, 3.3 eqv TBTU, 3.3 eqv. HOBt and 4.95 eqv. DIEA in NMP over night. The assembled product was cleaved from the resin with TFA-H_2O-triisopropylsilane (90:5:5, vol/vol/vol) to give Fmoc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHis-Leu-NH_2)$_2$ also denoted Fmoc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ as the major product according to MS. MALDI TOF MS: Calc. for $C_{144}H_{195}N_{37}O_{30}$, 2924.4; found 2923.6.

EXAMPLE 10

Synthesis of Fmoc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$, also denoted Fmoc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ (AGA-3-derivative of angiotensin-I amide, Fmoc-3-AGA synthesis of Seq. ID 5)

Product: Fmoc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Fmoc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ [LPA IX]

The peptide sequence AspArgValTyrIleHisProPheHisLeu (Seq. ID 5) was assembled on TentaGel S RAM resin (AM-linker) using standard coupling procedures with 3 eq. DIC and 3 eq. HOAt as coupling agent in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and coupled with 0.55 Fmoc-AGA, 3 eqv. PyAOP, 6 eqv. DIEA in DCM/NMP (3:1 vol/vol) dissolving Fmoc-AGA in NMP, PyAOP (activating

agent) in DCM and adding DIEA. Coupling over night. The assembled product was cleaved from the resin with TFA-H_2O-triisopropylsilane (90:5:5, vol/vol/vol) to give Fmoc-NHCH(CH_2CO-AspArgValTyrIleHis-ProPheHisLeu-NH_2)$_2$ also denoted Fmoc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ as the major product according to MS. MALDI TOF MS: Calc. for $C_{144}H_{195}N_{37}O_{30}$, 2924.4; found 2920.8. The product may be further purified by preparative HPLC or [Gel] gel filtration if desired. Figure 12 shows the MALDI-TOF MS spectrum for the crude product. Figure 13 shows the HPLC chromatogram for the crude product.

EXAMPLE 11

Synthesis of Aloc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Aloc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ (AGA-3-derivative of angiotensin-I amide, Fmoc-β-Ala and Dde-β-Ala synthesis of Seq. ID 5 using orthogonal protection of the sequence to be assembled)

Product: Aloc-NHCH(CH_2CO-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Aloc-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ [LPA-X]

The peptide sequence AspArgValTyrIleHisProPheHisLeu (Seq. ID 5) was assembled on TentaGel S RAM resin using standard coupling procedures with 3 eq DIC and 3 eq. HOAt as coupling agents in DMF. The N-terminal Fmoc-α-amino group was deprotected with piperidine-DMF and subsequently coupled with a mixture of Fmoc-β-Ala and Dde-β-Ala to give an orthogonal 1:1 Fmoc/Dde-protection of the N-terminus resin-peptide chains. The Fmoc-protecting group was deprotected with 20% piperidine-DMF followed by over night coupling with excess Aloc-3-AGA (6 eq.) and DIEA (12 eq.) in NMP. The remaining N-terminal Dde-α-amino groups were deprotected with 2% hydrazine in NMP for 10 minutes, followed by washing with 5% DIEA in NMP. Assembly was then achieved over night by activation with 6 eq. PyAOP and 12 eq. DIEA in NMP. The resulting product was cleaved from the resin with TFA-

H_2O-TIS (92.5:5:2.5, vol/vol/vol). MALDI TOF MS: Calc. for $C_{139}H_{199}N_{39}O_{32}$, 2928.6; found 2927.5.

EXAMPLE 12

Synthesis of Fmoc-AspProThrGlnAsnIleProProGly-NHCH(CH_2CO-AspArgVal-TyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Fmoc-Seq. ID 6-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ (AGA-3-derivative of angiotensin-I amide extended with Chlostridium thermosaccharolyticum peptide sequence 19-27)

Product: Fmoc-AspProThrGlnAsnIleProProGly-NHCH(CH_2CO-AspArg-ValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted Fmoc-Seq. ID 6-NHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ [LPA XI]

The Chlostridium thermosaccharolyticum peptide sequence 19-27 AspProThrGlnAsnIleProProGly (Seq. ID 6) was synthesised on chlorotrityl resin using standard peptide synthesis and cleaved from the resin with TFA-DCM (1:99, vol/vol) affording the fragment Fmoc-Asp(tBu)ProThr[-](tBu)Gln(Trt)Asn(Trt)IleProProGly-OH also denoted Fmoc-Seq. ID 11-OH. FmocNHCH(CH_2CO-AspArgValTyr[-]IleHisPro-PheHisLeu-NH_2)$_2$ also denoted FmocNHCH(CH_2CO-Seq. ID 5-NH_2)$_2$ was assembled as above (Example 11) and coupled with 3 eq. of the fragment using 3 eq. PyAOP and 6 eq. DIEA as coupling agents in NMP over night. The resulting fragment coupled product was cleaved from the resin with [TFA-H2O-TIS] TFA-H_2O-TIS (90:5:5, vol/vol/vol) to give the target compound together with some $NH_2CH(CH_2CO$-AspArgValTyrIleHisProPheHisLeu-NH_2)$_2$ also denoted $NH_2CH(CH_2CO$-Seq. ID 5-NH_2)$_2$. MALDI TOF MS: Calc. for $C_{184}H_{254}N_{46}O_{46}$, 3846.3; found 3842.8.

EXAMPLE 13

The performance of an ELISA based in LPA-I.

The capability of LPA-I according to the present invention to react with sera from patients with neuroborreliosis was evaluated against that of an ELISA based on biotinylated-pepC10, wherein pepC10 is